STUDIES ON NEUROMUSCULAR TRANSMISSION IN LIMULUS

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Among invertebrates only certain crustaceans and insects have been the subjects of detailed study in regard to neuromuscular mechanisms. There have proved to be very considerable differences between the various arthropod mechanisms encountered on the one hand (Wiersma, 1957—Crustacea; Hoyle, 1957—insects) and those of vertebrates on the other (Fatt, 1954). The differences contribute to the difficulty in arriving at a general concept of the way in which coupling between excitation of the surface membrane of the muscle fiber, which is achieved by nervous action, and shortening of the contractile material, is brought about. But they also show that certain favored hypotheses in regard to vertebrate muscle are either of only limited applicability for muscle as a whole, or are wide of the mark. There is a strong difference of opinion regarding the relevance of the electrical activity of the muscle fiber membrane in the process. Most recent authors (*cf.* Sten-Knudsen, 1954; Huxley, 1956) have regarded the contractile machinery as being in some way connected with the membrane potential. Some (*e.g.*, Csapo and Suzuki, 1957) believe that contraction is initiated by current flow resulting from membrane action potentials. For the Crustacea, it has been found necessary to postulate a separate coupling mechanism within the muscle fiber which may be activated differently by neuromuscular transmitter action in different cases. In some (Hoyle and Wiersma, 1958b) there may be a direct action by the transmitter substance on the coupling mechanism, the electrical intermediate (or propagation) stage having been by-passed. In others, electrical changes, or the ionic fluxes associated with them, affect the coupling mechanism.

From this it seems likely that in the elucidation of the general problems of excitation-contraction coupling, the arthropods will provide favorable material. In them single muscle fibers are innervated by more than one motor axon, each having different motor effects, and in the Crustacea there are also inhibitory axons which uncouple the excitatory action. In many arthropod systems the unit of contraction is not an all-or-nothing twitch, and contractions are minutely graded. This difference between arthropod muscle and ordinary skeletal muscle of vertebrates is probably attributable to the absence of propagated muscle action potentials in the former. In spite of their potential interest, and the variety of their mechanisms, several major subdivisions of the phylum remain unexplored, no arachnid, for example, having been examined in regard to its detailed neuromuscular mechanisms. It seems desirable, therefore, to have information regarding the motor mechanisms of the particularly interesting primitive arachnids, the Xiphosura. Accordingly a preliminary study has been made on *Limulus polyphemus* Latr. and has revealed several interesting features which are reported here.

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Methods

The walking legs, except the specialized fifth pair, have been examined from specimens 16″–22″ long, obtained at Woods Hole, with a view to finding suitable nerve-muscle preparations. The legs were severed by a quick snip of the coxo-trochanteral joint. A few of the leg muscles can be used, in particular the closer of the claw (adductor or depressor of the tarsus) and the flexor (levator) tibiae (situated in the patella). The present studies were carried out entirely on the claw closer. This muscle exhibits in the freshly-excised leg a remarkable pseudo-reflex. If the inside of the pollex (fixed extension of the tibia) is gently stroked, the claw closes sharply. This reflex can be obtained repeatedly for up to 15 minutes or so after removal of the leg. It seems highly improbable that any nervous machinery of true synaptic type can be present in the isolated leg to account for this curious phenomenon. Similar phenomena have been described

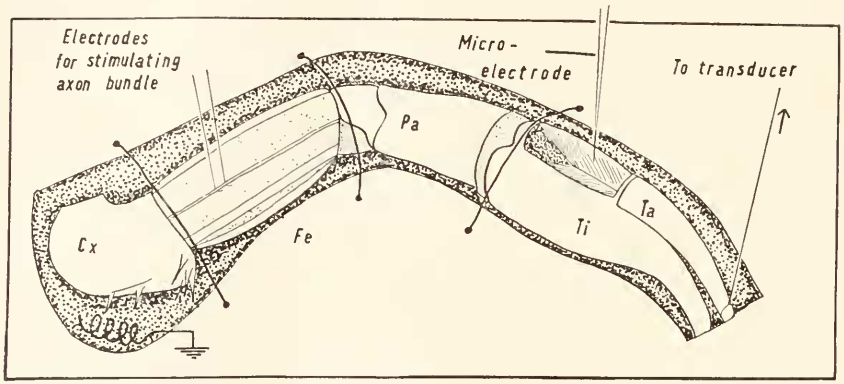

Figure 1. Drawing of the preparation seen from above. The leg is placed in a trough cut in a wax block. The opener of the claw has been removed, exposing the closer muscle.

in excised crustacean legs in which stretching of the chela, for example, can lead to its opening. Wiersma (unpublished) has suggested as an explanation of the crustacean responses that following excision the excitability of the cut ends of the motor nerves is raised to such an extent that an ephaptic transmission occurs from adjacent sensory axons.

To make a preparation, the excised leg was laid in a sculptured trough of wax and stapled into position with the tarsus uppermost. The main leg nerve can then be easily exposed in the femur by cutting away the shell and removing part of the extensor patella muscle. The nerve has no surrounding sheath and very little connective tissue so that it can easily be split into bundles. These may be stimulated in turn and any having an effect on the tarsus retained, the rest being cut away. The retained bundles can then be split again until either very small bundles, or eventually single axons, remain.

In this way it was ascertained that the closers of the claws of legs I–IV are innervated by two motor axons. No inhibitory axons were found. In this respect *Limulus* resembles the insects rather than the crustaceans.

There is no tested physiological saline for *Limulus* so filtered sea water was

used to bathe the preparation. Cole (1940) has analyzed the haemolymph and found that the mineral composition approximates very closely indeed that of the local sea water in two different localities, one of which was Woods Hole. Since the present work was done, a physiological saline has been developed for the Japanese horseshoe crab, *Tachypleus tridentatus* (Kikuchi and Tanaka, 1957).

At this stage a strip of shell was carefully snipped away from the margin of the tibia in order to expose the outer edge of the opener muscle (abductor tarsi). The opener apodeme was then cut close to the tarsus, grasped with forceps, lifted and stretched until the whole muscle came away. This leaves the V-shaped closer muscle exposed, with its innervation intact.

The pollex was fixed in a hole in the wax block and the tip of the tarsus was attached by a thread to an electromechanical transducer. Pairs of fine silver wires were micromanipulated onto the exposed nerve bundles. A drawing of the preparation, seen from above, is presented in Figure 1.

The muscle fibers are of fairly uniform diameter but only 25–40 μ thick, *i.e.*, they are appreciably thinner than many insect muscle fibers and very much thinner than those of the larger decapod crustaceans. Glass capillary micro-electrodes, filled with 3 *M* KCl, were used to record trans-membrane potentials from muscle fibers of the claw closer. The nerve bundles were stimulated with brief rectangular pulses isolated by radiofrequency coupling units. Display was conventional.

Results

In the more vigorous preparations a single stimulus applied to either of the two nerve fibers evokes in each case a small twitch. Repetitive stimuli lead to partial and complete tetani. The mechanical response to one of the two axons is, however, always larger than the other, and at a given frequency of stimulation also appears slower. Hence the two axons may be referred to as "fast" and "slow" according to the nature of the contraction evoked, as is customary in deal-

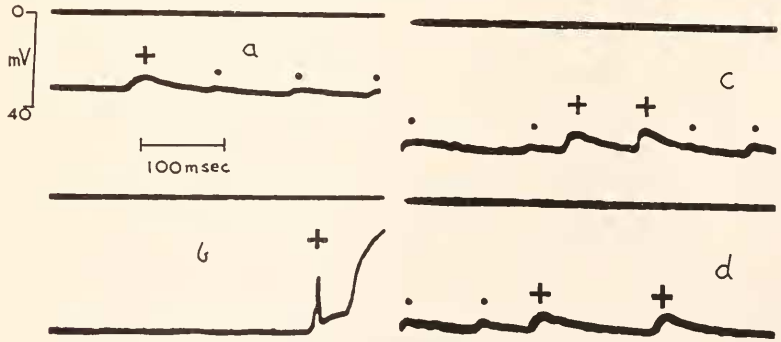

Figure 2. "Spontaneous" potentials. Four records from claw-closer muscle fibers of a fresh-excised *Limulus* leg, showing recurring potentials. The deflections marked + are attributed to discharges in the fast axon; those marked · to the slow axon. The single spike response in b was associated with a twitch which must have caused the electrode to be jerked out of the fiber. The upper trace in each record marks the zero baseline and the lower one the internal potential recorded with a 3 *M* KCl-filled glass capillary micro-electrode.

ing with crustacean motor nerve fibers (Wiersma, 1941). The corresponding responses are then called fast and slow, respectively.

"Spontaneous" responses. When the preparation is very fresh, discharges originating in the hypersensitive cut ends of the axons lead to spontaneous "tone" in the closer muscle and contractions which cause small movements of the tarsus. If a micro-electrode is inserted at random into a muscle fiber of the closer at this time, recurring electrical potentials of small size are seen (Fig. 2). The resting potentials of the muscle fibers are of small magnitude, ranging from 35–55 mV. The peak amplitudes of the recurring potentials are from 0.5 mV to a maximum of 25 mV in different fibers. In any one fiber they are clearly of two distinct sizes, the smaller being due to the slow axon and the larger to the fast. Single small potentials are not usually associated with visible twitches although in the more vigorous preparations, when they occurred singly, this was the case, and twitches were seen. A small proportion of muscle fibers gave "fast" potentials which were compound, *i.e.*, they had an initial component resembling an ordinary end-plate-potential (e.p.p.) giving rise to a small spike response (Fig. 2b).

The slow responses. Responses attributable to the "slow" axon could be observed in about 60% of those muscle fibers in which any appreciable electrical

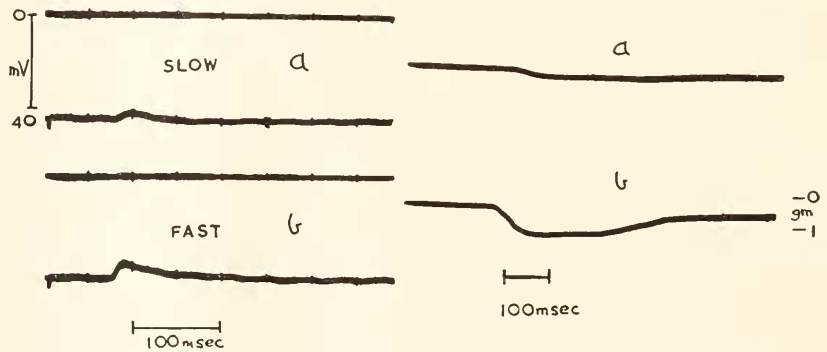

FIGURE 3. Potentials and tension due to single excitations applied to: a, the slow axon and b, the fast axon. Left hand side: electrical responses from the same single muscle fiber. Right hand side: mechanical responses of whole muscle recorded at tarsal tip.

change could be obtained during stimulation of both fast and slow axons (usually the bundles containing them) together. The single electrical response was always a very small one resembling a small e.p.p. It will be referred to as a junctional potential (j.p.) rather than an e.p.p. since nothing is known of the nature of the nerve terminals in *Limulus* muscle. To distinguish it from the corresponding response to the "fast" axon it will be called a slow junctional potential (s.j.p.) The long latency following the stimulus artifact, which is apparent in the records, is due largely to the conduction time of the nerve impulse along the nerve in the femur and patella into the tibia.

The s.j.p.'s rise to a peak in 12–18 msec. and decay in about 60 msec. The largest one found had a peak amplitude of 5 mV. Although no tension was usually recorded at the tip of the tarsus during stimulation of the slow axon with a single shock, some preparations did show a small twitch, giving not more than

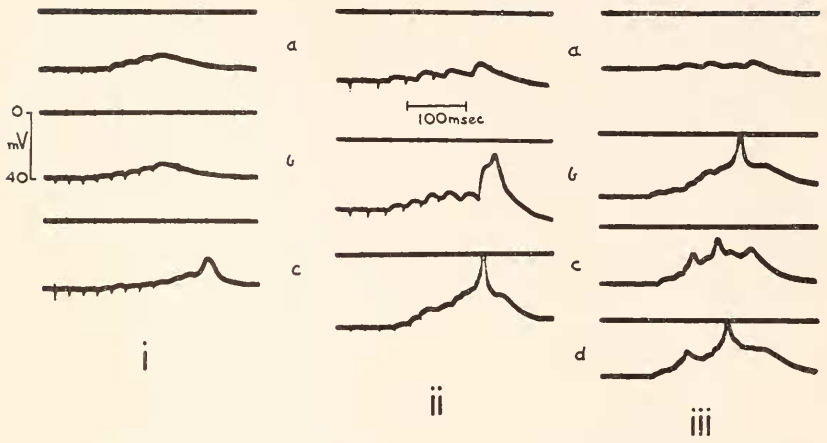

FIGURE 4. The responses to short trains of stimuli in three different muscle fibers (i–iii). (i) Slow axon. Three responses from the same fiber showing the summation of s.j.p.'s and the small degree of facilitation. A small spike arises in c from the plateau of depolarization. (ii and iii) Fast axon. a, low frequency; b–d, higher frequency. Successive steps (f.j.p.'s) are progressively larger (facilitation). Summation is evident; occasional spikes arise from the depolarization plateau.

0.5 gm. tension at the tip of the tibia (Fig. 3). On repetitive stimulation appreciable tension developed, increasing with increasing frequency of stimulation up to a maximum of just over 50 gm. at 200 per second. Thus the tetanus/twitch ratio was more than 100: 1. The s.j.p.'s initially increased in magnitude by two or three times during a train of stimulation, a phenomenon usually referred to as facilitation, but later diminished as they also summated to give a plateau of depolarization. From the plateau occasionally a small spike arises (Fig. 4i, c).

The fast response. The fast axon evoked electrical responses in most of the muscle fibers penetrated. They were often very small, but they were always larger

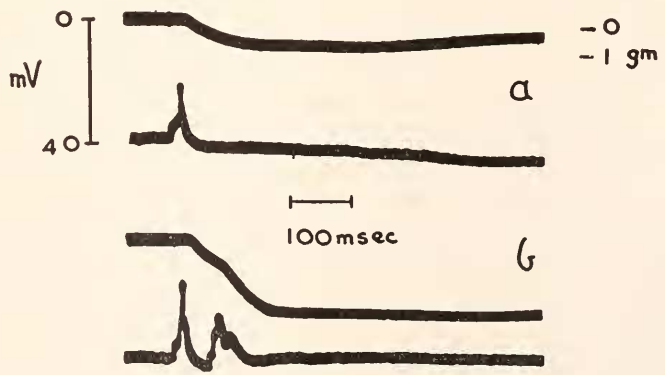

FIGURE 5. Electrical response of one fiber and total mechanical response at tip of tarsus to: single (a) and paired (b) stimulation of the fast axon. Note that the f.j.p. is followed by a small spike in each case.

than the corresponding slow responses, when these were seen, in the same fibers. There was no overlap of s.j.p. and f.j.p. magnitudes in individual fibers, such as was found in several muscles of decapod crustaceans (Hoyle and Wiersma, 1958a). The typical response to a single shock is shown in Figure 2. The response, like the s.j.p., is of end-plate-potential type and will be referred to as the fast junctional potential (f.j.p.). The rise-time of the f.j.p.'s was usually about the same as that of the s.j.p.'s, *i.e.*, 12–18 msec. and the decay likewise about 60 msec. But occasionally an f.j.p. had a faster rise-time of only 5–6 msec. and/or a faster decay of about 40 msec. In some fibers the f.j.p. leads to a small spike of 10–15 mV. The larger f.j.p.'s reached a peak amplitude of 11 mV.

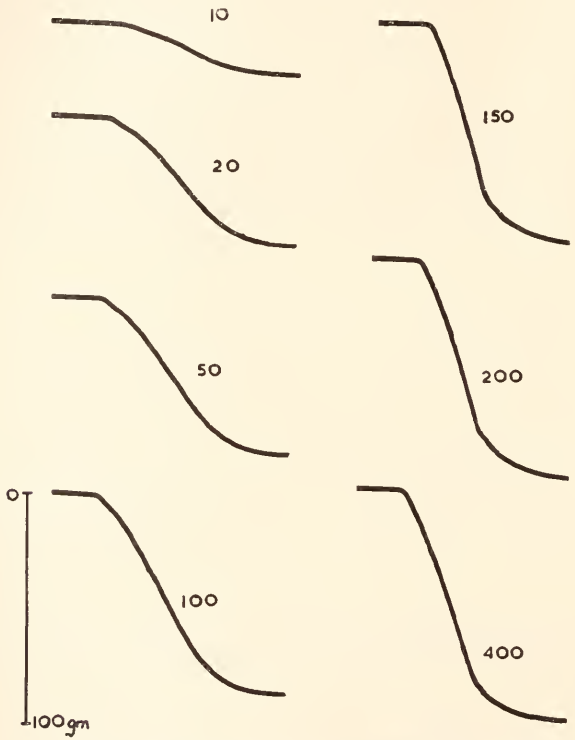

FIGURE 6. Tension recorded during stimulation of the fast axon to show the development of tetanus at the various frequencies indicated.

When pairs of shocks are applied to the axon the mechanical responses summate and also show facilitation (Fig. 5) as the interval between the shocks is reduced. If the first f.j.p. evokes a spike then the second one, at intervals up to 200 msec., seldom does so or gives a much smaller one, *i.e.*, there is a long relatively refractory period for the spike. If the first f.j.p. is of relatively large size but does not give a spike then the second usually evokes one. When the paired shocks are applied regularly, at a low repetition rate, the character of the response is seen to change from time to time. Thus the first f.j.p. will soon fail to evoke

a spike but the second will lead to one and vice versa, the process being reversed again after a while. The spike mechanism either fatigues very easily or it is a very labile response.

With prolonged repetitive stimulation, whether there is spiking or not, a plateau of depolarization builds up and is maintained. Brief bursts of stimulation illustrate the way in which the plateau builds up (Fig. 4ii, b–d). At the higher frequencies spikes, taking off from the depolarization plateau, may just reach and occasionally overshoot the zero baseline (Fig. 4i, c). The total tetanus tension and also the rate of rise of tension, continue to increase with increasing frequency of stimulation up to a maximum at about 200 per second (Fig. 6). The tetanus tension measured at the tip of the tarsus then exceeds 100 gm. The tetanus/twitch ratio is ordinarily about 30:1 but it increases as the preparation ages, eventually becoming infinite as the twitch response just fails.

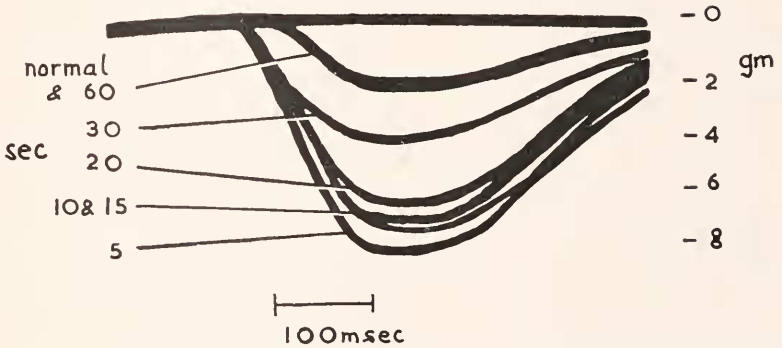

FIGURE 7. Records of tension developed in response to a single shock applied at various intervals (as indicated in seconds) after a brief tetanus (100 shocks at 100/sec.).

The electromechanical transducer used was a fluid potentiometer. When lightly-loaded it recorded the twitch tensions associated with the slow axon of 0.5 gm. and less. Under these loading conditions the tension records did not have the usual shape for a twitch but instead showed a plateau of tension. This may have been caused in part by sluggishness of the potentiometer, but the plateau was too long to be due entirely to this. Thus, in the absence of a large restoring force (the twitch is normal in appearance with a spring attached to the load), tension is maintained for about half a second, after which relaxation occurs.

Post-tetanic potentiation. Following a very brief tetanus there is an enormous potentiation of the twitch response which is regularly 5 times, and may be as much as 7 times, greater than the normal twitch tension. The effect subsides gradually over a period of 45–60 seconds (Fig. 7). The intracellular leads showed no electrical concomitant of this enhancement in the individual muscle fibers examined. It would of course be necessary to examine a large number, particularly in respect to increased tendency to give spikes, in order to be sure that there was no significant electrical effect and this has not been attempted. In the fibers examined the f.j.p.'s were facilitated following the tetanus but only for a few seconds, a fraction of the time during which the tension is potentiated.

Discussion

From the electrical activity recorded in various muscle fibers of the closer of the claw of the walking leg of *Limulus* it may be inferred that the pattern of innervation is substantially similar to that found in doubly-innervated crustacean muscles and non-specialized insect muscles. That is, most of the muscle fibers are themselves innervated by both slow and fast motor axons (polyneural innervation). It has not been established in this investigation that the innervation is also multi-terminal, as it is in those insects and crustaceans which have been examined closely, *i.e.*, that the axons make synapse with the muscle fibers at several points along their length.

The rather low resting potentials and small size of the electrical responses might suggest that the muscles deteriorate following excision of the limb. But for periods up to two hours in which the preparation was used there usually was no (further?) decline in their value. Thereafter, decline was fairly rapid. Tetanus tension measured at the tarsal tip in the preparation is at least as great as that which can be obtained by evoking reflex closure of the claw in the intact animal.

Both the s.j.p.'s and the f.j.p.'s differ in peak amplitude in different fibers although the fast is always larger than the slow. Their rise and decay times have not been determined critically in the present experiments, partly because they were somewhat variable. In some fibers the fast response had both a faster rise time and a faster decay time than the slow, but this was not often encountered and in most cases they had similar values. There was no evidence of a "paradox" situation similar to that found in certain Crustacea (Hoyle and Wiersma, 1958b); *i.e.*, the slow axon did not give tension at lower frequencies of excitation than those which just failed for the fast. There was, in fact, unlike the situation in many crustaceans (Hoyle and Wiersma, 1958a) nothing to indicate that the slow and fast transmitter substances need be regarded as qualitatively different chemically. The preliminary results could be interpreted on the basis of quantitatively different extents of release of one transmitter substance from the terminals of the fast and slow axons.

Each junctional potential attains a constant height over long periods of intermittent stimulation, but the secondary, small spike responses are extremely unpredictable in their appearance and magnitude. They arise only from the larger f.j.p.'s or from the plateaux of depolarization in tetanus. But they cannot be said to appear at a particular level of membrane potential. They may be present on one occasion and absent on the next although the same j.p. deflection is reached in both. Also, they occur randomly, not synchronously, in the population of fibers so that it cannot be determined whether or not their appearance leads to extra tension.

Facilitation of junctional potentials is present in both fast and slow systems, quite markedly in some fibers, hardly at all in others. It is more marked than it appears in the records. The long time-course ensures that there is summation even at low frequencies of stimulation. Hence the later j.p.'s in a train appear at lower and lower levels of membrane potential. Since the magnitude of a j.p. is proportional to the magnitude of the membrane potential, they thus appear quite a bit smaller than they would if the same amount of transmitter action occurred at the normal resting potential level.

The total tension is related to the extent of maintained depolarization, in the randomly-selected muscle fibers studied, during tetanus at different frequencies. But the enhanced tension which occurs in the period following shortly after a tetanus is not reflected in increased depolarization. This argues against there being a simple causal connection between membrane potential and tension. There is probably a connection between the strong post-tetanic potentiation and the fact that there is a high tetanus/twitch ratio, but both must be attributed to intra-muscle-fiber events rather than to neuromuscular junctional ones. All these facts make it seem probable that further and more detailed investigations of neuromuscular transmission in *Limulus* will make valuable contributions to our understanding of excitation-contraction coupling in muscle.

I wish to thank Professor H. Grundfest for the generous facilities which he placed at my disposal in his laboratory at Woods Hole.

Summary

1. The electrical responses occurring in single muscle fibers of the closer muscles of the chelae of the walking legs of *Limulus* have been studied with the aid of intracellular electrodes and electrical stimulation of the motor axons. At the same time the total tension of the muscle was recorded at the tarsal tip.

2. The muscle is supplied by only two motor nerve fibers, one of which (the "fast" axon) evokes larger mechanical and electrical responses than does the other (the "slow" axon).

3. No inhibitory nerve fiber was found.

4. The electrical responses consist typically of junctional potentials resembling small end-plate potentials. The fast junctional potentials may give rise to small spike potentials.

5. On repetitive stimulation both axons give rise to plateaux of depolarization, from which small spikes may arise.

6. The mechanical responses consist of very small twitches to single shocks and tetani to repetitive excitation. The tetanus/twitch ratio is more than 30 : 1 for the fast axon, more than 100 : 1 for the slow axon.

7. There is post-tetanic potentiation of the twitch response of up to 5 times in the mechanical response to a single shock applied to the fast axon. This decays slowly over a period of about a minute.

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